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Trends in Counter-Current Chromatography: Applications to Natural Products Purification

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Counter-current chromatography (CCC), a unique continuous liquid-liquid partition chromatographic technique, has been widely used in the separation and purification of natural products. This technique has made great progress in recent years. In this paper, the different possible separation methods developed in CCC are described. It includes classical CCC, pH-zone-refining, pH-gradient, ion-pairing, multi-channel and two-dimensional CCC, dual-flow, liquid-liquid-liquid three-phase systems, elution-extrusion, gradient elution, powder direct injection, with some words for the various techniques used in pre- and post-CCC procedures, and detection modes. The basic mechanisms of each method are presented and applications are summarized. The future of CCC developments, including its application in protein purification and enrichment, and new platforms, are also discussed. This review paper aims to aid scientists who are interested in the field of CCC research for natural product isolation.

KEYWORDS Counter-current chromatography, natural products, separation and purification

INTRODUCTION

Modern analytical chemistry (AC) has the difficult task to generate data needed in almost all aspects of the chemical world. Separation is the third

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most important field in AC just after weighting (balances) and electrochemical methods (pH controls). Chromatographic techniques include high-performance liquid chromatography (HPLC), gas chromatography (GC), supercritical fluid chromatography (SFC), capillary electrophoresis (CE), thin-layer chromatography (TLC) and counter-current chromatography (CCC). Counter-current chromatography (CCC) is a developing technique based on the principle of liquid-liquid partition. The novelty of this technique is that it uses only liquid phases to perform a separation. Both the mobile phase and the stationary phase are liquids. Two different designs allows for establishing a liquid stationary phase: the hydrostatic design and the hydrodynamic design (1). Since the vast majority of natural product purifications were performed using hydrodynamic CCC equipments, for clarity, the hydrostatic design and apparatuses will just be rapidly evoked at the end of this review.

CCC takes full advantage of the liquid nature of the stationary phase. Compared with conventional column chromatography, it eliminates the complications resulting from the solid support matrix, such as irreversible adsorptive sample loss, stationary phase deactivation, tailing of solute peaks and contamination (2, 3). It also allows a sample load range from micrograms to kilograms depending on the column size (4). In addition, the solvent system used in CCC can be almost any combination of solvents producing two liquid phases. This covers an enormous number of possible combinations. CCC often has good resolution and reproducibility (3).

In the past three decades, a number of publications on the theories, principles, designs, practical applications and the technical details of CCC have been reported. The CCC technique is now accepted as an efficient preparative as well as analytical technique and can be coupled with many types of detectors for the qualitative and quantitative analysis of various natural products (5, 6). The application of this technique to separate natural compounds in traditional Chinese medicines (TCMs) has developed into a major area in its application field. Most TCMs are a mixture of an extremely large number of small molecules with different molecular weights, structure classes and hydrophobicity. The width of the hydrophobicity window and sample loading are all significantly challenging for the CCC technique. A long separation time is needed, due to the narrow hydrophobicity range for a single solvent system, in the isocratic elution mode. Improvements of solvent retention, extension of the hydrophobicity windows, and scaling-up the significant sample loads have became major themes in CCC development.

Numerous studies for dealing with these problems have been published recently and many applications have been used to illustrate and validate the feasibility of the use of CCC. For ionizable compounds, such as alkaloids and organic acids, pH- dependent CCC techniques such as pH-zone-refining and ion-pairing have been employed (7, 8).

Elution-extrusion (EECCC) and back-extrusion (BECCC) can extend the hydrophobicity windows to more solutes, i.e., having partition coefficients or distribution constants, K_D, going practically from zero to infinity (9, 10). For the insufficient separation of structural analogues of TCMs, the emergence of multi- channel (MC-CCC), two-dimensional (2D-CCC) and liquid-liquid-liquid three-phase (LLL-TP-CCC) were proposed to further improve the resolution capabilities with compounds in a complex matrix (11–13).

For simple separations, the power direct injection (PDI-CCC) method is the most promising development, and is suitable for separating the components present in plant powdered extracts by simply dissolving the extract in the two-phase system (14). For complex extracts, different elution methods can be used to improve the resolution power significantly. They are: classical isocratic elution, gradient elution (with possible flow gradient, step-gradient, polar gradient and pH gradient) and the two dual elution modes (15–19). These different CCC modes can be used for various vegetal species and complex active components of the TCMs. Thus, the content determination and fingerprint analysis of TCMs can be performed efficiently by CCC for a variety of active components (20, 21).

Apart from the separating and analyzing methods mentioned above, various techniques used in pre- and post-CCC procedures are often required, which are critically important for successful purification and analysis. Conventional liquid- liquid extraction, silica gel, ionic sephadex and macroporous size exclusion resin column chromatography are techniques commonly used before the CCC step to pre-purify the sample. In general, following these optimized pre-purification steps that eliminate the unwanted sample components, pure compounds can often be separated by a one-step run. However, often impure fractions are produced after a single run, especially when the sample is very complex. Then, further purification of the fractions is required, and various techniques including preparative liquid chromatography, re-crystallization, conventional column chromatography or even a second CCC purification, are used.

Detection in CCC is critical. On-line detection is desired, so hyphenated CCC techniques have been developed. The three main types of hyphenation are: CCC-UV/ELSD (Evaporative Light Scattering Detector), CCC-HPLC-DAD (Diode Array Detector) and CCC-MS (Mass Spectrometry).

In this paper, different CCC separation methods of natural products including classical CCC, pH-zone-refining, pH-gradient, Ion Pairing, dual CCC, 2D-CCC, MC-CCC, LLL-TP-CCC, elution-extrusion, gradient and Powder Direct Injection, coupling techniques as well as pre- and post-CCC processes, and on-line coupling techniques, are all reviewed. The schematic contents are shown in Figure 1. In addition, the future of CCC developments is tentatively evoked.

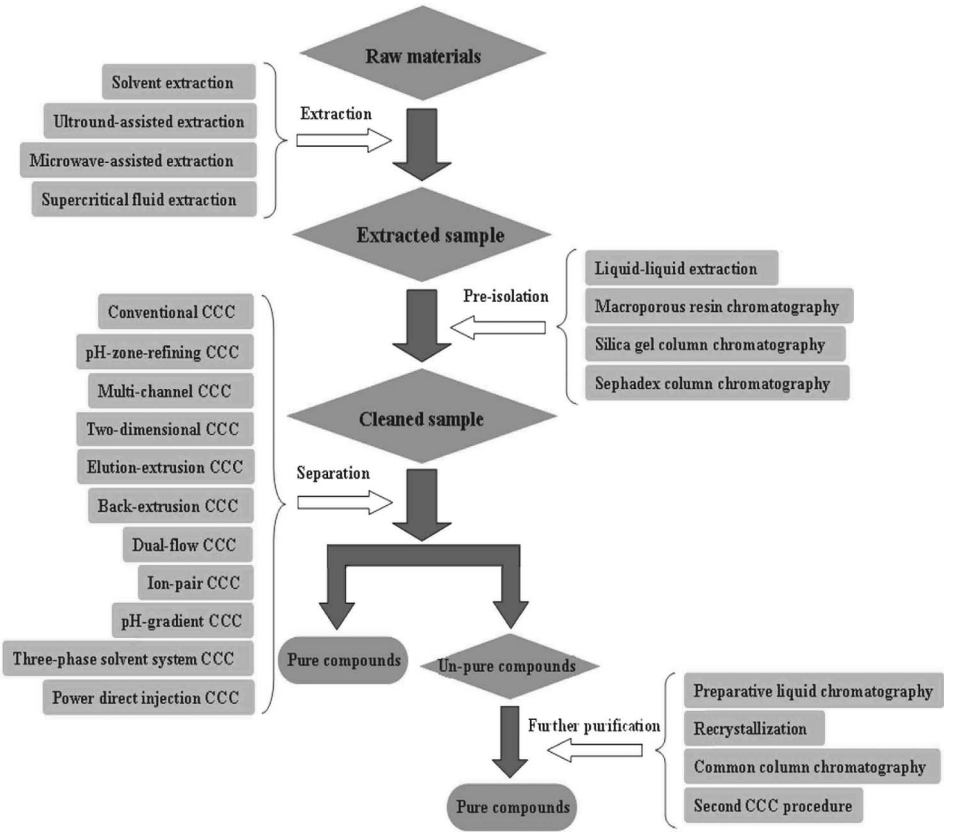

FIGURE 1 The strategy of purification and analysis for the nature products, including four sections of extraction, pre-isolation, separation and further purification.

MAIN CCC SEPARATION METHODS

Classical CCC

The standard hydrodynamic CCC apparatus uses a type-J multilayer coil planet centrifuge design, schematically illustrated by Figure 2. A bobbin is filled by coiling an open tube in multi layers on it. A planetary motion of the coil is obtained using two rotation axes and the gear arrangement shown in the figure. The two major benefits of this arrangement are 1) a rotary-seal-free elution system is obtained, and 2) a unique hydrodynamic motion of the biphasic liquid system is created inside the multilayer coil. This CCC design was developed by Dr. Yoichiro Ito in the 1980's. Since then, it has been widely used in the separation and analytical fields. It is widely used in the separation of natural products and fingerprint analysis of TCMs (22, 23).

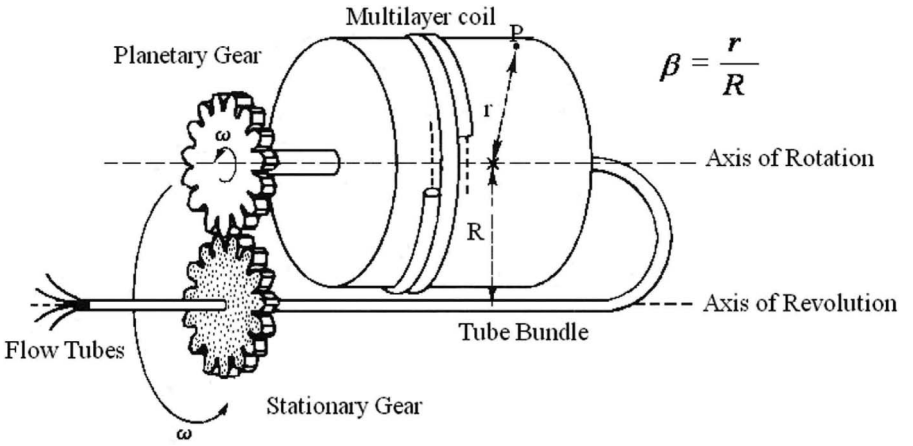

FIGURE 2 Type-J multilayer coil planet centrifuge of the standard hydrodynamic. The multilayer coil separation column holder revolves around its own axis, when it is rotating about the axis at the same direction and angular velocity [Adapted from Ref. (2)]. Reprinted with permission of Elsevier.

The CCC "column" (i.e., the apparatus) is first filled with the stationary phase. The column rotation is started and the mobile phase is introduced in the right direction: the descending or head-to-tail direction if the mobile phase is the lower (denser) phase of the biphasic system and oppositely, the ascending or head-to-tail direction if the mobile phase is the lighter upper phase (3, 9, 20). The mobile phase displaces a volume of stationary phase that is measured collecting it at the column outlet. When the mobile phase is seen exiting at the column outlet, the liquid-liquid equilibrium is reached and the displaced stationary phase volume collected correspond to V_m, the volume of mobile phase inside the column of total volume, V_C. The volume of liquid stationary phase retained inside the column is:

$$V_s = V_C - V_m \tag{1}$$

The stationary phase relative retention ratio, *Sf*, is defined as:

$$Sf = V_s / V_C \tag{2}$$

Any solute injected in the equilibrated column will be eluted after a volume, V_r, of mobile phase has been passed:

$$V_r = V_m + K\ V_s \tag{3}$$

In which K is the solute distribution ratio between the two phases. K is also called solute partition coefficient ($K = C_s/C_m$, where C_s is the concentration of

the solute in all its chemical forms in the stationary phase, and C_m is the concentration of all forms of the solute in the mobile phase). The quality of the Compound 1 (K_1) and Compound 2 (K_2) separation is measured by the resolution factor, *Rs*, expressed by (9):

$$Rs = Sf \frac{\sqrt{N}}{4} \frac{K_2 - K_1}{1 - Sf[1 - (K_2 + K_1)/2]} \quad (4)$$

in which N is the number of theoretical plates of the CCC column. Eq. 4 shows that the resolution factor depends directly on *Sf*, the amount of liquid stationary phase retained in the CCC column.

The selection of the solvent system is also an important factor for a successful separation since it will act critically on the solute K values. Many publications appeared explaining how to optimize the solvent choice (24, 25). The two best methods are: 1) literature is searched for CCC separations of similar chemical structures or identical compounds and the liquid system is used again, 2) solvent series such as the ones developed by Ito, Oka and the useful alkane/ethyl acetate/methanol water series are tested.

The mixture of test compounds called GUESSmix can greatly help to select the right solvent system (26). Of course, the solute partition coefficients, K, can be directly determined in each phases of the biphasic liquid system using HPLC, TLC, GC or other spectroscopic mean. The suitable solvent system can be selected according to the K values, namely when $K <$ 0.5, the elution time is short (eq. 3) and the resolution between peaks can be reduced (eq. 4). When $K > 2$, the solute elution time is long and the peaks become broad. Thus, the solvent system producing adequate K values in the range of 0.5 to 2 is recommended.

Column temperature has a significant effect on K values, the retention of the stationary phase and the mutual solubility of the two-phase system. High temperature also produces a decreased fluid viscosity. Generally, a higher revolution speed will produce a better liquid stationary phase *Sf* and will be associated to a better phase mixing producing an enhanced efficiency, N, hence a better resolution *Rs* (eq. 4). High centrifugal field equipments are desired, however, such equipments require high standard in term of mechanical construction and balancing. Analytical CCC apparatuses have lower volume and lower rotating masses, they will be easier to build following the mechanical requirement and have high rotation capabilities generating high field. Preparative CCC instruments are often rotated at a low speed due to their size and mass. High centrifugal fields allow for higher flow rates since the liquid stationary phase is tightly held. High flow rates of the mobile phase result in shorter run time and better productivity.

Conversely, low flow rates result in lengthy separation duration that can still produce purified compounds with a reduced productivity. Liu *et al.* have separated and purified five hydroxyanthraquinones and cinnamic acid from the Chinese medicinal herb *Rheum officinale* Baill by hydrodynamic CCC. HPLC and CCC chromatograms of crude extract from *R. officinale* Baill are shown in Figure 3 (27). Six peaks with high purity were obtained and the separation yielded 19 mg of peak I, 19 mg of peak II, 18 mg of peak III, 14 mg of peak IV, 10 mg of peak V and 6 mg of peak VI from 120 mg of the crude extract.

Conventionally, the sample is dissolved in the mobile phase or the stationary phase or both phases. When the sample size is small, it can be

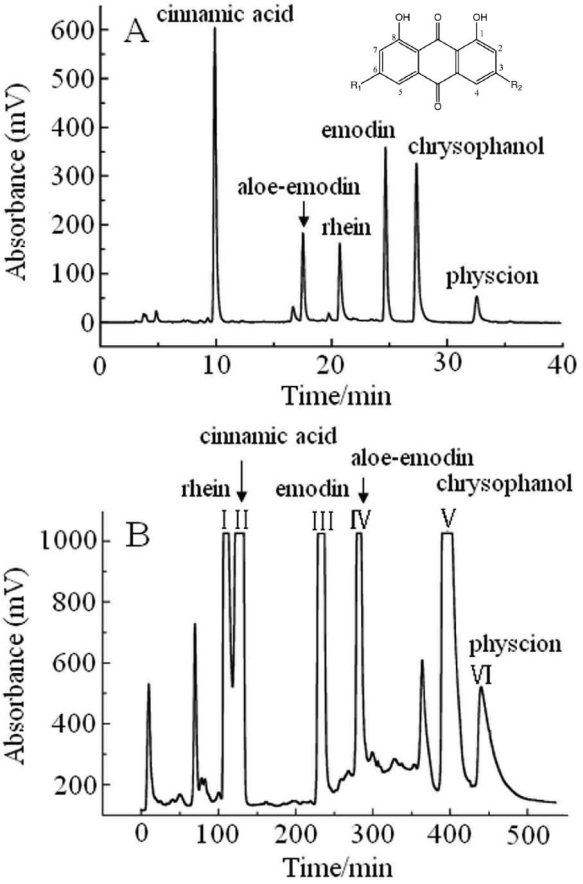

FIGURE 3 HPLC (30 min) and hydrodynamic CCC chromatograms of crude extract from *R. officinale* Baill. (A) HPLC chromatogram of the extract on Spherigel ODS 25×0.46 cm, gradient elution from 57% to 90% methanol in pH 1 phosphoric acid; (B) Slow CCC chromatogram (9 hours) with diethylether/pH gradient buffer, column 260 mL, extract size 120 mg, 800 rpm, 2 mL/min buffer mobile phase in the descending head-to-tail direction. (Adapted from Ref. [27]). Reprinted with permission from Elsevier.

dissolved easily. When the sample size is large, suspension solutions with particles can be injected into the column, inducing sometimes a stationary phase loss. Thus, the loadable sample size must be considered based on the column size, the apparatus performance and the solubility of the sample in the solvent system.

In order to improve the retention of the liquid stationary phase of organic- aqueous and two-aqueous solvent systems, a multiple spiral disk assembly for type-J CCC was recently developed (28). Acceptable resolutions were achieved when different solvent systems and different elution modes were used (29, 30). Although hydrodynamic CCC has been widely used and developed in the separation and analysis of natural products, some micro- and multi-component samples, strong acids or strong base chemicals can not be purified very well. Other CCC techniques should be used, such as pH-zone-refining CCC, MC-CCC, 2D-CCC and EECCC.

pH-Zone-Refining CCC

pH-zone-refining CCC is a special separation method using the fact that chemical reactions are possible with a liquid stationary phase. The separating process of pH-zone-refining involves the titration of the basic stationary phase by the acidic mobile phase (or vice versa). Ionizable compounds injected when the titration starts will arrange themselves in bands sorted by their ionization constant of pK_a. An on-line pH meter detection system must be used to detect the change in pH value during the procedure (31).

According to the different mobile phases, pH-zone-refining has two modes. One is normal displacement mode, and the other is reverse displacement mode. The former uses an organic phase as the mobile phase and the polar aqueous phase as the stationary phase. The eluting acidic reagent in the mobile phase protonates the ionized analytes, displacing them in their molecular form from the aqueous stationary phase toward the organic mobile phase, and vice versa in the normal displacement mode. The analytes elute in bands of similar heights. The normal mode eluting sequence is reversed in the reverse displacement mode (32).

The partition coefficient, K, of the retained acids is a factor that must be considered with the pK_A to understand the band positions. K is the value of the partition coefficient of the retained acid; K_A is the ionisable acid dissociation constant, more commonly used as the log value expressed by the pK_A. The acid K value is a measure of its tendency to go in the organic phase in its molecular form. The acid pK_A value is a measure of its tendency to ionize upon pH changes. The ionized form of the acid has a greater affinity for the aqueous phase. There are always several acidic compounds separated in pH zone refining. In certain cases, a compound 1 very a high hydrophobicity (high K_1) value associated to a high dissociation constant (low pK_{A1}) may produce a band, which can overlap with a Compound 2 that would have

both lower hydrophobicity and lower dissociation constant. Neat bands are obtained when hydrophobicity and dissociation constant are correctly matched which is often obtained working with high concentration of retainer base and displacer acid (33).

pH-zone-refining can use a variety of retainer acids as compatible agents to separate the mixture of ionisable compounds. Compared to traditional CCC, pH-zone- refining can separate highly polar ionisable compounds such as amino acid derivatives, peptide derivatives, proteins, alkaloids, acids and enantiomers with large sample size (34, 35). Zheng et al. (36) separated three alkaloids from *Nelumbo nucifera* leaves by conventional and pH-zone-refining CCC, and the CCC chromatograms of the two separations and HPLC chromatograms of each obtained fractions are all shown in Figure 4. With conventional CCC, they purified ~3 mg of A, 28 mg of B and ~2 mg of C with the purity of 99.0%, 98.7% and 98.1% after an injection of 120 mg of a crude extract. With pH-zone-refining CCC, they obtained 120 mg of A, 1020 mg of B and 96 mg of C after an injection of 4.0 g of a crude extract, with the purity of 98.7%, 99.0% and 98.0%.

Comparing the separation effective and sample-loading capacity of two modes, the results clearly demonstrated that pH-zone refining CCC has a more than 10-fold increase potential in sample-loading capacity, high purity, and high concentration of collected fractions. In addition, according to the pH value of the successive eluted fractions, the band position can be detected accurately, even when the analyte has no spectral absorption.

However, pH-zone-refining also has some limitations. The analyte must be ionisable, and the difference between solute dissociation constants (pK_A) should be at least 0.2 unit. Furthermore, the technique requires significant sample concentration, and it does not work well at low concentration. The present method may be used to purify various other alkaloids and acids from nature products.

Ion-Pairing in CCC (IP)

Organic acids and alkaloids are always hydrophilic compounds with good solubility in water, methanol, ethanol and their mixtures. These compounds are not always efficiently separated by CCC using conventional solvent systems, due to the low retention of solutes in the stationary phase. Therefore, finding a suitable CCC solvent system is a challenge. In order to extend the hydrophobicity window, a novel CCC solvent system has been developed. Low amounts of specific acids or bases are added to the solvent system to ionize the compounds and form ion-pairs. The ion-paired solutes have a much lower polarity than the original ions. Ion-pairs have a completely different solubility and partitioning behavior in the biphasic liquid system. Hence, a different and better resolution can be achieved (9, 37).

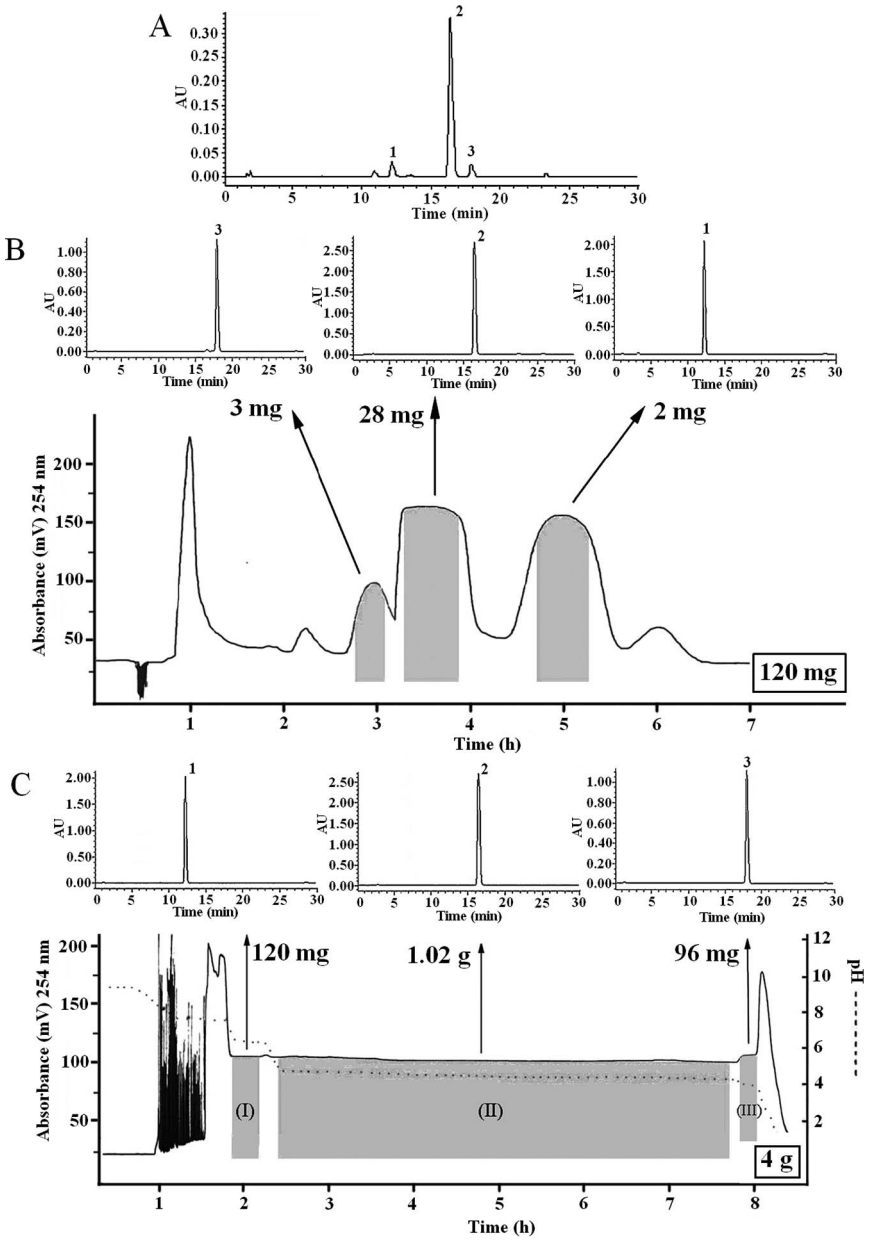

FIGURE 4 The results of HPLC analyses and pH-zone-refining CCC separation of alkaloids from *Nelumbo nucifera* leaves. (A) HPLC chromatogram of alkaloid extract; (B) Classical slow CCC separation (7 hours) and HPLC control for the purification of 120 mg of alkaloid extract (system chloroform/methanol/water 4/3/2), flow rate ascending tail-to-head aqueous phase, 800 rpm, CCC column 230 mL); (C) pH-Zone-refining (petroleum ether/ethyl acetate/methanol/water 5/5/2/8 v/v with 0.01 M triethylamine in the upper organic phase and 0.005 M HCl in the lower aqueous phase, 1.5 ml/min descending head-to-tail, 800 rpm, CCC column 230 mL) and HPLC control for the separation of 4.0 g of alkaloid extract. [Adapted from Ref. (36)]. Reprinted with permission of Elsevier.

IP is highly suitable for the preparative isolation and analysis of very polar and unstable compounds. The advantages of using ion-pair substances are that they facilitate the isolation and determination of minor concentrated components using a single chromatographic technique, as all derivatives of the targets when adequately paired are less polar and have better solubility in the organic stationary phase of the solvent system (higher *K* values). However, the CCC separation method frequently leads to different elution orders of the chemicals when compared to similar ion-pairing HPLC analysis. In order to increase the solubility of the compounds in the organic phase, suitable ion-pairing reagents are required and are selected according to the physicochemical properties of the components. The ion-pairing agent must have a high purity, water solubility, UV transparency up to 200 nm and volatility since the recovered compound must be freed from it (38).

Jerz et al. first prepared betalain pigments by means of IP-CCC from berry extracts of *Phytolacca americana* (Phytolaccaceae). The CCC chromatogram and the HPLC analysis of fractions are shown in Figure 5.

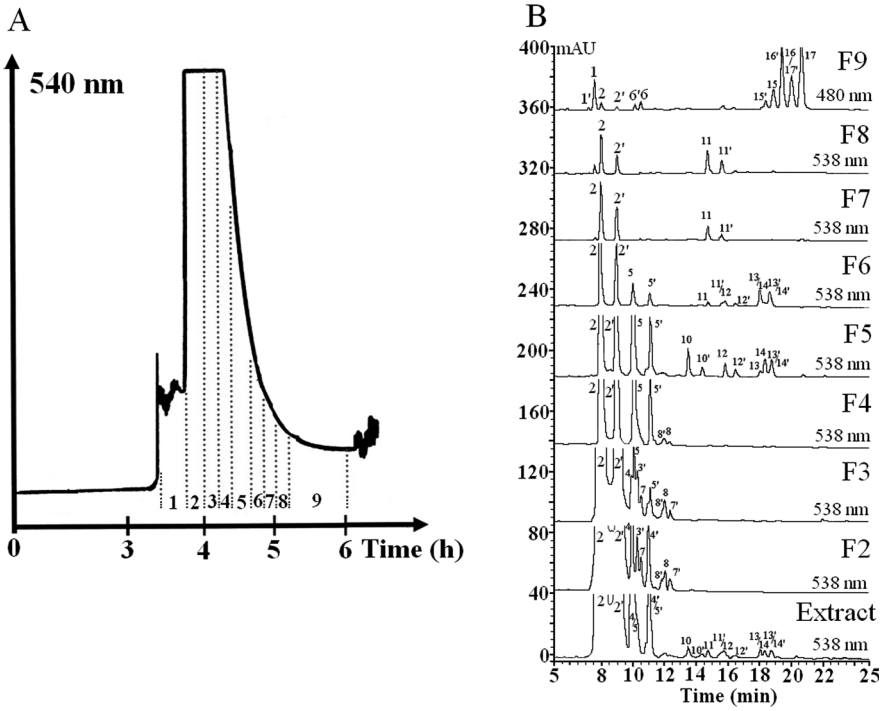

FIGURE 5 The results of separation of C_{18}-enriched pigment extract (900 mg) of berries *Phytolacca Americana*. (A) Ion-pair CCC chromatogram (BuOH/ACN/water with 0.7% trifluoroacetic acid pairing agent 5/1/6 v/v, acidic mobile phase 3 mL/min descending head-to-tail mode, 850 mL CCC column, 850 rpm); (B) HPLC analysis of crude extract and the 9 obtained fractions. (Adapted from Ref [39]). Reprinted with permission of Elsevier.

Ion-pair forming trifluoroacetic acid (TFA) was added at low concentration (0.7%, v/v) in the aqueous lower phase of the solvent system consisting of 1-butanol–acetonitrile–water (5:1:6, v/v). The TFA ion-paired polar betacyanins and betaxanthins had a much higher affinity for the organic stationary phase. The resolution between the polar and less polar betacyanins is now satisfactory for the enrichment of the hydrophobic minor concentrated pigments for further studies (39). The experiment proved the IP-CCC is a complementary methodology to other preparative method.

In conclusion, IP has great potential for separation of acids and alkaloids from natural sources. However, the method for separating most polar compounds still requires improvement, and the resolution between polar and less polar compounds is not satisfactory for the enrichment of hydrophobic minor concentrated compounds for future studies.

Extrusion CCC

Extracts obtained from medicinal herbs used in TCMs contain an extremely large number of small molecules differing in molecular weight, structural class, and hydrophobicity. Classical CCC has a limited hydrophobic range and resolution power often associated with long separation time in isocratic elution mode. CCC is unable to analyze and separate natural products with a wide polarity range effectively. Hence extrusion CCC has been developed to extend the hydrophobicity window. Variation of the method was also proposed such as elution-extrusion (EECCC) and back-extrusion (BECCC) (40, 41).

Compared with classical CCC, elution-extrusion can extensively enhance the separation ability of any single biphasic liquid system and polarity range, and can be very useful when screening TCMs. Both EECCC and BECCC are performed using classical standard CCC centrifuges. The methods take full advantage of the liquid nature of the stationary phase by causing it to move through the coiled tubing. Band broadening inside the chromatography system depends on the band position, and the advantages of EECCC and BECCC rely on the narrow band widths present inside the column (42).

In the EECCC procedure, there are three steps: the first step is classical elution, where the column is first filled with the stationary phase, then the coils are rotated at the desired speed, and the mobile phase is pumped in at the selected flow rate in the correct direction (e.g., from head-to-tail if the mobile phase is the lower phase). When the hydrodynamic equilibrium is established, the sample is injected and the chromatogram is developed classically. This is the elution step. After at least a column volume of mobile phase is passed (so that all solute with K value lower than 1 are eluted, eq. 3), the mobile phase is simply replaced by the liquid stationary phase without changing neither the flow rate or direction nor the rotor rotation. This

produce the extrusion of the column content (solute with $K > 1$) maintaining the solute partial separation that was obtained inside the CCC column. After another column volume of liquid stationary phase has been pumped inside the CCC column, it is certain that all the injected material is extruded. Nothing can stay trapped inside the CCC equipment and everything injected is recovered (10).

The EECCC method allows for the rapid screening of numerous samples. It is extremely suitable for high-throughput separation. For example, EECCC was used to screen the crude ethanol extract of *Zingiber cassumunar* and to isolate milligram-amounts of bioactive components (43). The correlative chromatograms are shown in Figure 6. Due to potential difficulties in peak recognition caused by UV detection, a clarifying reagent was added post the column to smooth the UV-signal. The advantages of EECCC are obvious. The EECCC provides an extended sweet spot of separation and offers high-resolution separation of wider polarity range of analytes. At the

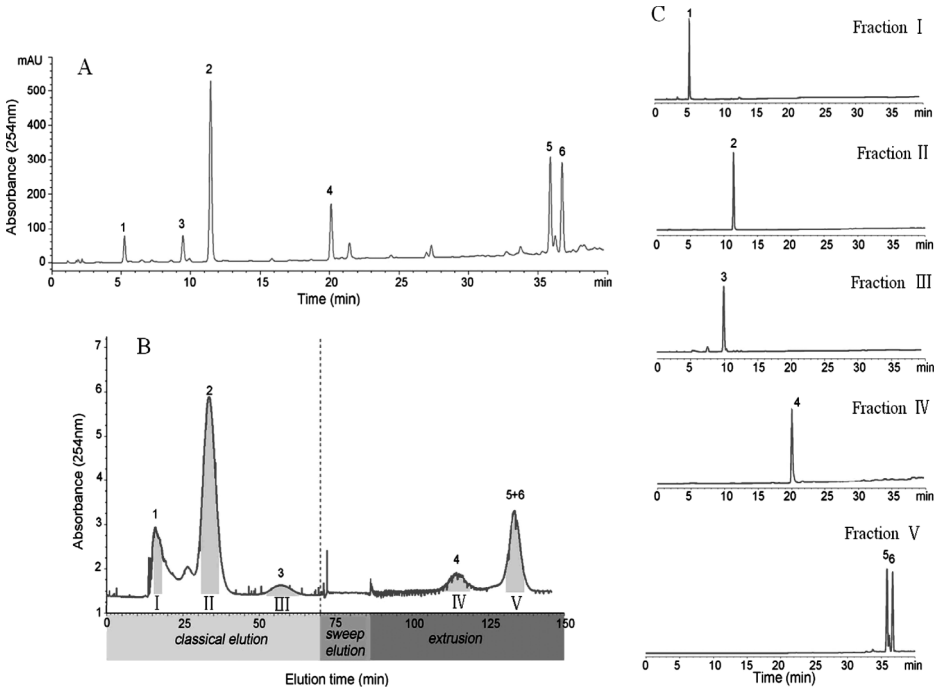

FIGURE 6 The results of separation of *Z. cassumunar*. (A) HPLC analysis of the crude ethanol extract; (B) EECCC screening of *Z. cassumunar* components. Classical elution for 70 min (140 mL with 2 mL/min of lower aqueous phase of the heptane/ethyl acetate/methanol/water 1/1/1/1 v/v system, 650 rpm), then extrusion pushing at 2 mL/min the upper organic phase of the biphasic liquid system. After another 70 min (140 mL) everything contained in the 280 mL CCC column is eluted; (C) HPLC analysis of the obtained fractions (Adapted from Ref [43]). Reprinted with permission of Elsevier.

end of the EECCC procedure, the CCC apparatus is full of stationary phase, ready for the next experiment.

A similar elution method called "dual-rotation elution" was also developed to increase the resolution power of CCC. This method used special equipment able to rotate in both directions. Forward-rotations were followed by counter-rotations. In the example given, the mobile phase (lower phase) was pumped in the head-to-tail direction when the CCC column was forward-rotated. After no peaks are eluted out, the upper phase was used as mobile phase entering in the same line because head and tail were switched by counter-rotating the CCC column (44). This particular dual-rotation elution could decrease the separation time and increase resolution.

Back extrusion (BECCC) was recently proposed as a very simple other way to use the liquid nature of the stationary phase. The first stage is again a classic elution. When there is sufficient mobile phase passed through the column in the right direction, say one column volume so that all solute with $K < 1$ are eluted (eq. 3), the first stage is completed. The second stage is back-extrusion. A 4-port valve is used and is switched to change the flow direction of the mobile phase to the opposite "wrong" direction. This flow direction change produces a sudden decantation of two liquid phases inside the column, and the parts of the solutes in the stationary phase are back extruded by the mobile phase with relatively sharp peaks. The parts of the solutes contained in the mobile phase during the classical elution step remain in the mobile phase forming small "ghost" peaks. The method is designed to save time and reach high recoveries of the retained solutes (10). Some comparisons of EECCC and BECCC have been made to demonstrate their usefulness (45).

pH-Gradient

The classical CCC method using neutral solvent systems for separation of organic acids and alkaloids does not always produce satisfactory results due to poor solute solubility in non-polar solvents. Thus, a new CCC method called pH-gradient with a hydrophilic solvent system containing high ratios of acids or bases has been developed, which is characterized by low interfacial tension**,** high viscosity, and a step wise change of mobile phase pH (19).

The pH-gradient method offers various advantages over classical CCC methods, such as the large sample capacity, high concentration of eluted fractions, efficient enrichment and detection of minor components present in a large quantity of the crude samples. Furthermore, the pH zone can be extended according to the character of the target compounds. This method can be used for the quantitation and fingerprint analysis of TCMs containing organic acids or alkaloids (46). It has been successfully applied in the separation of natural products including acidic and basic derivatives of amino acids, oligopeptides, hydroxyxanthene dye and alkaloids (47).

The pH-gradient system procedure is as follows: the multilayer coil is first filled with the upper organic stationary phase, which may be acidic, basic or neutral. The sample solution is then injected into the column, and two parts of the lower aqueous mobile phase with different pH values are pumped in the descending or 'head-to-tail' direction with the linear proportion gradient ratio. The change in volume ratios for the two mobile phase solution with different pHs is determined by the chemical properties of the target compounds, and peak fractions are collected according to the elution profile. This continuous pH-gradient elution mode can improve the mobile phase solute solubility and high resolution, purity and recovery can be obtained (46, 48).

Dual Continuous CCC

Standard CCC has more advantages than some other column chromatography techniques. However, some aspects of CCC still need to be improved. The main drawbacks of classical CCC are that it is time consuming and it may be difficult to find the appropriate solvent system. Also a concentrated injection can induce losses of liquid stationary phase and accumulation of interfering substances in the column. Continuous separation is desirable. Very early the dual CCC design was described and developed to reduce these problems (12).

The coil column of for dual CCC must be greatly adapted so that it is possible to continuously inject the sample in the middle of the coil and, at the same time enter one liquid phase on one side of the coil while collecting the other liquid phase on the same side of the coil. The modified coil had five flow channels, two for each terminal (inlet and outlet) and one in the middle for sample injection. With this special coil, it is possible to have the two phases of the solvent system moving through the coil in opposite directions to each other in a true countercurrent way. When a complex sample containing multiple compounds is injected in the middle of the coil, a number of its contained chemicals will be carried in one direction, while the rest will be carried in the opposite direction. Adapting the flows, it should be possible to select on which side compounds are collected. Both hydrophilic and hydrophobic compounds can be washed out simultaneously from the column (49). Continuous dual CCC was theoretically modeled and used in an effective coupling with ESI-MS/MS and NMR techniques (50, 51)

Two-Dimensional CCC

For the separation and analysis of natural product complex samples, one-dimensional chromatography is unsuitable. Thus, multi-dimensional chromatography has been developed, and includes two-dimensional gas chromatography (2D-GC), two-dimensional HPLC (2D-HPLC) and two-dimensional

CCC (2D-CCC). Multi- dimensional chromatography is the best strategy to provide much higher resolution, better recovery and peak capacity when compared with one-dimensional chromato- graphy (13).

As we know, the general CCC one-dimensional technique is problematic in the analysis and separation of some complex samples. When two or more compounds overlap in a CCC peak, some techniques including conventional column chromato- graphy, preparative liquid chromatography, or a second CCC run are often required to complete the purification. This is effective but tedious and time consuming. Thus, 2D-CCC has been studied (52).

The 2D-CCC separating system is composed of two different volume units connected by a column-switching system or commercial six-port valve. The first CCC column separate incompletely parts of the injected sample. When these incompletely purified fractions elute from the first CCC, they are introduced in the second CCC column running with a different liquid system. Full separation can be achieved this way. Some successful examples have been reported (53).

Multi-channel CCC (MC-CCC)

In classical CCC, the improved elution mode and apparatus have resulted in good retention of the stationary phase, large sample loading capacity, and good partition of target compounds. An original approach was to build a parallel CCC apparatus. The MC-CCC apparatus has been designed that has three independent coils forming three independent CCC columns in the same rotor (11). With parallel flow tubes, these columns will perform identical synchronous planetary motion but with possible different liquid systems. Three independent chromatographic runs can be carried out simultaneously. Thus, a high-throughput CCC fractionation method was developed to separate ethyl acetate extracts from three herbs by combining the use of the new three-channel CCC apparatus and conventional parallel chromatographic devices (54). In the three-channel CCC apparatus, three fractionation processes can be achieved, thus processing three times the amount of samples as a classical CCC system in a one-time procedure.

The MC-CCC is very useful for high throughput fractionation of natural products for drug discovery. However, an additional requirement is that the different types of solvent systems used in the different channels should be of similar density to balance the centrifuge system. Moreover, in some cases, especially when the common CCC apparatus is used in one-channel separation, this system can be improved to a multi-channel device by changing the connection of the flow tubes and not lengthening the PTFE tubes of the separation columns (55). This method enhances the throughput and shortens the separation time and will reduce the CCC resolution because of the shortened separation columns. Thus, there is work before these equipments can be marketed as useful and easy CCC columns.

Liquid-Liquid-Liquid Three-phase System (LLL-TP)

Some aqueous-organic solvent systems can form three phases at specific volume ratios, examples are: as *n*-hexane–methyl acetate–acetonitrile–water, *n*-hexane–ethyl acetate–acetonitrile–water and *n*-hexane–tertbutyl methyl ether–acetonitrile–water. The composition of the three-phase solvent system is selected according to the parameters of the solutions, such as volume ratio, kinematic viscosities and specific gravity of the upper, middle and lower phases. The kinematic viscosity of the mobile phase influences the retention of the stationary phase. The differences in specific gravities between the upper phase (UP) and the middle phase (MP), and the MP and the lower phase (LP) are easily measured. This density difference is related to the retention of the stationary phase. The solute partition coefficients are measured either between the UP and the MP or between the MP and the LP. For a single solute, the $K_{UP/MP}$ is used if its hydrophobicity is high; the $K_{MP/LP}$ will be preferred for hydrophilic compounds.

The three-phase system composed of *n*-hexane–methyl acetate–acetonitrile–water (4:4:3:4, v/v/v/v) was selected for the separation of a mixture of 15 standard compounds with a wide range in hydrophobicity from *β*-carotene to tryptophan (56). The chromatograms are shown in Figure 7. Then, the solvent system was used for compositional analysis of several crude natural drugs and tea products made by a different process to provide the useful information of the hydrophobic diversity of whole components present in each natural product.

The separation mechanism of the three-phase solvent system is identical to that of as the two-phase solvent system. The initial stationary phase loaded in the column is a equal mixture of MP and LP. The hydrophobic compounds are eluted first by the UP, and then the moderately hydrophobic compounds are washed out by the MP (Fig. 7). The most polar compounds still remaining in the column may be recovered from the column by extruding the LP. Thus, CCC used with a three-phase solvent system has some extended possibilities in the separation of complex samples such as raw extracts of natural compounds (13).

Gradient Elution

The isocratic elution mode is the preferred mode used in CCC since any change introduced in a phase of a biphasic liquid system will induce composition changes in the other liquid phase. In other words, it is not possible to perform gradient in CCC as easily as in HPLC since all composition changes of mobile phase also changes the stationary phase.

Stepwise gradient elution was performed preparing three different compositions of the same biphasic liquid system (*n*-hexane-ethyl acetate-methanol-water). Then the UP (stationary phase) and the LP (mobile phase)

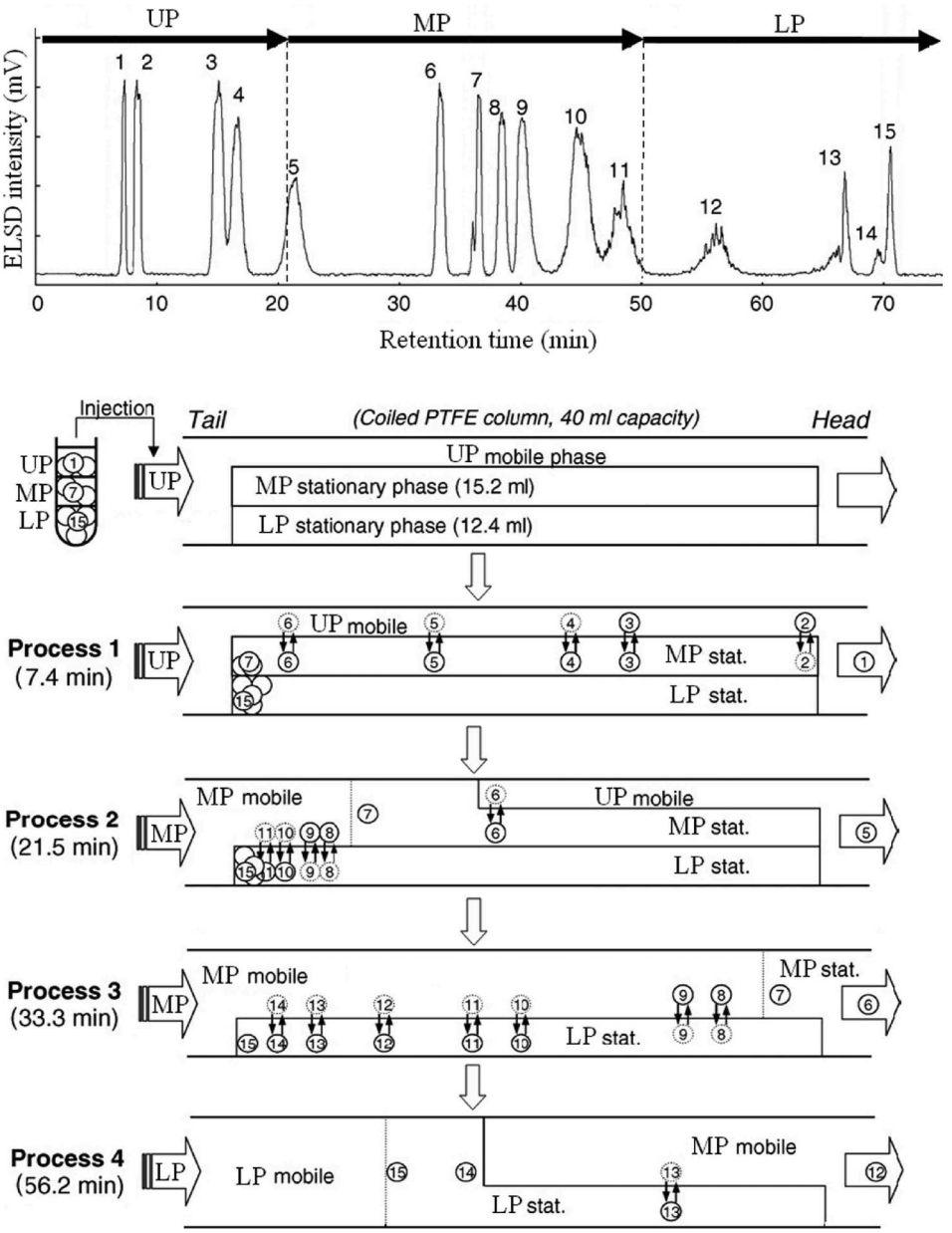

FIGURE 7 Characteristics of CCC separation using three-phase solvent system, hexane/methyl acetate/acetonitrile/water 4/4/3/4 v/v under optimized conditions. (A) The CCCl separation chromatogram of mixture of standard compounds; (B) CCC separation process of 15 standards showing the successive phase role and the 2 extrusion processes, 2 of the UP phase and 4 of the MP intermediate phase. [Adapted from Ref. (56)]. Reprinted with permission of Elsevier.

of the first composition system were pumped into a hydrodynamic CCC column. When the hydrodynamic equilibrium was reached, the sample solution was injected and the separation started. After some time, the UP of the first composition was changed for the UP of the second composition and finally the mobile phase was changed for the UP of the third composition. In this process, a large portion of the stationary phase (initially LP of the first composition) is lost by internal composition re-equilibration (16, 17). Reducing the flow rate of the mobile phase and increasing the revolution speed of the apparatus can improve the withholding of the stationary phase (57). Fine adjustments between flow rate and mobile phase composition can have beneficial effects (58).

Powder Direct Injection

The usual process for TCM extraction and CCC purification is 1-extraction of the powdered herbs by solvents and 2-extract concentration for injection in a CCC column. It would be ideal if the herb powders could be directly injected into the CCC column. The technique of powder direct injection (PDI) has some advantages including it is simple to perform, time- and solvent-saving, and has a potential high recovery of desired molecules. Four types of alkaloids from *Coptis chinensis* Franch were successfully obtained with high recoveries of over 92% by direct plant powder injection (14). The PDI method was also shown to be effective to obtain purified molecules from different plants. Rutin was obtained from powdered *Flos Sophorae* (59) and gentiopocroside from *Radix gentianae* (unpublished).

However, not all samples can be separated and analyzed in this way. Some parameters such as the content of the target in the raw material, the dissolution behavior of the two phases to the chemicals in the plant powder, the size of the powder particles and their swelling capacity in the CCC column must be considered. Some problems can occur with this technique, such column clogging, sample running off with the mobile phase, and dramatic changes in liquid stationary phase retention volume.

SAMPLE PREPARATION

If the PDI method is not appropriate, the sample must be prepared before the CCC purification. Most often two steps are required: extraction and cleaning-up. The extraction step is performed using either solvent extraction, Soxhlet extraction, ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE) or supercritical fluid extraction (SFE). The shortcomings of extraction steps are 1-the time needed, 2-solvent consumption and 3-possible sample decomposition caused by heat or intense ultrasonic vibrations (UAE). MAE based on the intensive and quick heating

effect of microwaves can overcome the problems of conventional solvent extraction (60–62).

SFE uses supercritical CO_2 fluid as an extractant. Compared with the other methods mentioned, SFE has an efficient extraction effect with the virtue of being a green technique using no organic solvents. However, SFE is most suitable for the extraction of liposoluble constituents, and some polar chemicals can also be extracted using this technique by adding modifiers to the medicinal herbs (63).

The extract must be cleaned up. Liquid-liquid extraction (LLE), conventional column chromatography (silica gel, polyamide and Sephadex), and macroporous resin chromatography are extensively used. Among these techniques, LLE and conventional column chromatography are simple. But they need significant amount of expensive solvents. Macroporous resin is a type of solid adsorbent that is cheap, has a wide application, large adsorption capacity and sample loading, and can be used more than 1,000 times. More importantly, aqueous ethanol can be used to elute the compounds. Macroporous resin has been widely used in the cleaning-up of natural product extracts (64).

Post-CCC Processes

CCC has a great separating power but the natural products extracts are often so complex than the CCC fractions need further purification to be useful. Traditional column chromatography (flash LC) is able to purify the CCC fractions. In LC, three types of solid adsorbents are used: polyamide, bare silica gel and Sephadex ion exchange resin (65). Recrystallization is another technique able to increase the purity of CCC fractions. The process is based on the difference in solubility of the solutes. The separated solutes are concentrated so that a saturated solution is obtained. The impure compounds stay in solution when the desired compound precipitate.

Recrystallization is difficult with numerous parameters to adjust: temperature, temperature gradient, crystal seeding, time and energy, stirring, over-saturation problems. Done properly, recrystallization produces crystals with a high purity and a low sample loss (66). Preparative LC (prep LC) is another LC method used to purify CCC fractions. However, the expensive Prep LC system is sometimes necessary, but has not been widely used in common laboratories since a second CCC run can often do the job (67). When apparatus and conditions are limited, a second CCC step is often used with a different liquid system. The two CCC steps are effective, valid, workable and widely used (68).

Detectors

Initially, the CCC effluent was collected in fraction that was one by one checked by different methods. Nowadays, on-line techniques for detection

have been developed mainly with UV detectors. Evaporative light scattering detectors (ELSD), mass spectrometers (MS) even with the powerful MS/MS technique and diode array detectors (DAD) are also used. On-line purity determination or identification can remove additional steps in the off-line post-separation and decrease the processing time.

Generally, a UV detector with a single or multi-wavelength is the most widely used for on-line detection, but the chemicals should show strong UV adsorption. The ELSD is very useful in CCC since it evaporates the eluent eliminating spikes seen with a spectroscopic UV detector due to microdroplets of stationary phase commonly present in the mobile phase (69). However, the ELSD can detect only non-volatile solutes. This is, fortunately, the case for many components of natural products. Recently the charged aerosol detector (CAD) was introduced by the ESA-Dionex company and could well be useful in CCC detection. The DAD is a spectroscopic detector very sensitive to the stationary phase microdroplets; however, it is able to monitor a large spectrum at the same time, allowing for some identification of UV adsorbing components (70).

MS is the technique of choice for solute identification. It coupling with CCC poses little problem just needing a split on the CCC column outlet since the MS cannot accept the high flow rates commonly used in CCC (71). Structure elucidation of the natural products in multi-component crude extracts is possible when MS/MS is used (72). However, there are also drawbacks with this on-line technique. The instrumentation of MS is not popularly used as it is very expensive, and the effluent from the CCC column is not always stable.

Other exotic on-line techniques were used with the CCC column. For example, on-line radical scavenging detection by use of 2,2′-diphenyl-1-picryl hydrazyle (DPPH) as model radical was successfully used for preparative isolation and screening of antioxidant components from an ethyl acetate extract of the bacteria *Selaginella moellendorffii* (73).

Protein Separation and Enrichment

Due to the wide application and research of proteins in the fields of foodstuffs, nourishment, medicine, medical diagnosis and biocatalysis, the development of new purification techniques and the enrichment of proteins are critically important in protein chemistry, proteomics, biotechnology and pharmaceutical science. Some chromatographic techniques, including ion exchange chromatography (IEC), gel chromatography, capillary electrochromatography (CEC), RP-HPLC and affinity membrane chromatography have been developed and are widely used in protein science (74).

Aqueous two-phase liquid systems (ATPS) were very early used in CCC to purify proteins. They are made by a water solution of phosphate salts and polyethylene glycol (PEG) or dextran (75). However, ATPS are very difficult to retain in a hydrodynamic CCC column, X-axis apparatuses were not very reliable from a mechanical point of view, so the newly developed

spiral disk assembly seems promising for protein purification (76). To further improve the efficiency of the spiral disk, some modifications of the channel configuration were made.

These modifications included the channel configuration being divided into multiple round partition units or compartments, which were serially connected with narrow transfer ducts (77); a mixer-settler spiral disk which alternately mixes and settles the polymer phases in multiple pairs of two elongated sections divided by barricades (78); and the original spiral tube assembly being improved by flat-twisted tubing or changing the shape of the tubing, which can interrupt the laminar flow of the mobile phase (79, 80).

Reverse micelle solvent systems were recently proposed. They have been used for separating proteins using liquid-liquid partition techniques in a separating funnel, such as dioleyl phosphoric acid (DOLPA)-isooctane and cetyl trimethyl ammonium bromide (CTAB)-isooctane, and trioctylmethylammonium chloride (TOMAC) - isooctane (81, 82). A protein mixture consisting of myoglobin, cytochrome *c*, and lysozyme has been successfully separated by CCC using a reverse micelle solvent system, KCl-containing buffer solution/diethylhexyl sufosuccinate (AOT)-containing hexane. Separation efficiency was significantly improved by adjusted pH and ionic strength gradient elution, and the chromatogram and the elution curves of each protein are shown in Figure 8. Protein recovery in the total fractions was 83% for myoglobin, 90% for cytochrome *c*, and 82% for lysozyme (83). The feasibility of simultaneous separation and enrichment of protein demonstrated that this technique may have potential in the separation and enrichment of biomacromolecules in the future.

Hydrostatic CCC

Hydrostatic CCC (centrifugal partition chromatography, CPC) is a particular kind of liquid-liquid counter-current chromatography technique. In the machines, there is a single axis of rotation, columns use channels interconnected by ducts in series, mounted in the centrifuge rotor. It also offers capabilities for the retention of the liquid stationary phase in the column via a strong and constant centrifugal field. During CPC separation, the different elution modes, such as gradient elution, ion-exchange displacement and pH-zone refine mode, can be applied to improve the separation efficiency and total separation time (84–86).

CPC can reach a good separation using both organic–aqueous and aqueous–aqueous two-phase systems for the separation. It can be said that CPC columns have very similar uses than classical hydrodynamic CCC columns with a simpler mechanical design (a single rotation axis versus two for hydrodynamic column). However, CPC columns work at a much higher pressure (between 0.5 and 8 Mpa or 5 and 80 kg/cm^2) than hydrodynamic columns working at pressures lower than 0.5 MPa. CPC apparatuses have

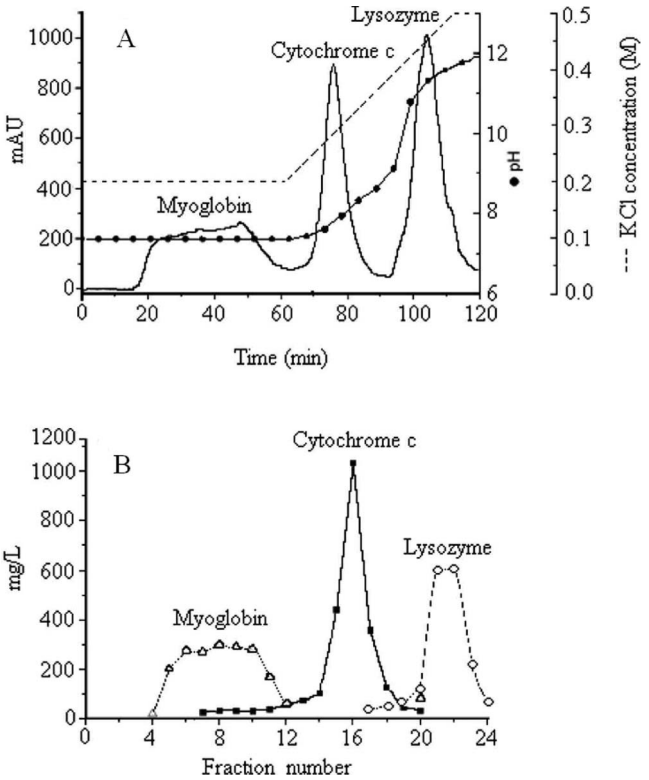

FIGURE 8 Protein separation and enrichment using pH and KCl concentration gradient elution. (A) CCC chromatogram obtained with a 38 mL column and the liquid system stationary phase hexane (with 0.1 M sodium diethylhexyl sulfosuccinate) and 1 mL/min initial mobile phase buffer (0.05 M tris-HCl pH 7 plus 0.2 M KCl and final buffer (0.05 M phosphate pH 12, 0.5 M KCl), sample injection: 1.5 mg, on-line UV detector monitored at 280 nm; pH measured in each 5 mL fraction; KCl concentration computed using the fractions of mobile phases A and B; (B) protein concentrations in the 25 collected fractions acquired by HPLC analysis. [Adapted from Ref (83)]. Reprinted with permission of Elsevier.

been used to perform efficient separations of various classes of natural products, involving flavonoids, saponins, coumarins, anthraquinones, phenolic acids and naphthoquinones (87). They also have been successfully applied to a number of analytes including human serum proteins, recombinant ketosteroid isomerase, carotenoid cleavage enzymes, plasmid DNA, polysaccharide, polymerized pigments, PEG-protein conjugates, etc.

CONCLUSION

CCC has found its use in the purification of components found in natural products and especially in the extraction of active principles in traditional Chinese medicines. The fact that CCC uses a liquid stationary phase is a big

advantage that greatly compensates for the big problem of its retention in rotor-containing columns. The recent release of reliable modern hydrodynamic CCC centrifuges is making the work easier. The separation proponents have always been reluctant to use unfamiliar hydrostatic CCC columns. Considering the great savings in solvent volumes and solid stationary phases and the significant throughput of the technique, the presence of CCC columns for rapid purification in the pharmaceutical, food and organic synthesis industries will continue to increase in the near future.

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